Isolation and Purification of the Designer Drug Metabolite *O*-Desethyl-*N*-(1-phenylcyclohexyl)-3-ethoxypropanamine (*O*-Desethyl-PCEPA) Biotechnologically Synthesized using Fission Yeast Expressing CYP2D6

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Abstract

Reference standards of drug metabolites are needed for their structural confirmation and pharmacologic/toxicologic characterization, but such metabolite standards are often not commercially available. Biotechnological synthesis of such metabolites using human CYP2D6 heterologously expressed in fission yeast *Schizosaccharomyces pombe* has recently proved feasible for the model drug 4'-methyl-α-pyrrolidinobutyrophenone and its 4'-hydroxymethyl metabolite (FT Peters et al., TIAFT 2006). The aim of the present study was to apply this new approach to synthesize the *O*-deethyl metabolite of the designer drug *N*-(1-phenylcyclohexyl)-3-ethoxypropanamine (PCEPA). For synthesis of *O*-deethyl-PCEPA, 74 mg of PCEPA·HCl were incubated with 1 L of CAD58 culture (a *S. pombe* strain expressing human CYP2D6) for 72 h. After centrifugation, the supernatant was brought to pH 3 with conc. H_3PO_4 and submitted to solid-phase extraction (SPE) using Varian Bond Elut SCX HF cartridges (5 g, 20 ml). The eluate was evaporated to dryness and reconstituted in 3 mL HPLC solvent. Aliquots (250 µL) were separated by semi-preparative HPLC [Merck LiChrospher® RP select column, 250×25 mm, 5µm; 50 mmol/L ammonium formate buffer (pH 3.5)/acetonitril (80:20 v/v), 5 ml/min; UV detection at 263 nm]. The eluent fractions corresponding to the metabolite were collected, diluted with water (1:4 v/v) and submitted to SPE as described above. From the eluate, *O*-deethyl-PCEPA was isolated and analyzed by GC-MS, ^{1}H-NMR, HPLC. PCEPA was only partially metabolized by the heterologously expressed enzymes. *O*-deethyl-PCEPA and the remaining parent drug were effectively extracted from the incubation supernatants by SPE. *O*-deethyl-PCEPA could be separated from the remaining parent drug and from matrix compounds within 30 min. SPE also proved efficient for isolation of the metabolite from the collected eluent fractions. The identity and structure of the product were confirmed by GC-MS and ^{1}H-NMR. The purity was found to be greater than 90% as determined by HPLC.

1. Introduction

Reference standards of drug metabolites are needed for their structural confirmation and pharmacologic and toxicologic characterization. However, such metabolite standards are often not commercially available, particularly in the case

of new therapeutic drugs or drugs of abuse. The classical chemical synthesis of drug metabolites can be cumbersome and stereochemically demanding, while custom made synthesis is often a time-consuming and very expensive solution.

Recently, Peters et al. [1] demonstrated that biotechnological synthesis of drug metabolites using cytochrome P450 (CYP) enzymes can be a versatile alternative to classical chemical synthesis. They used the pyrrolidinophenone-type designer drug 4'-methyl-α-pyrrolidinobutyrophenone (MPBP) as model substrate and human cytochrome P450 (CYP) 2D6 heterologously expressed in strain CAD58 of the fission yeast *Schizosaccharomyces pombe* as model enzyme. After 48 h incubation of 250 µmol of MPBP, the MPBP metabolite 4'-hydroxymethyl-α-pyrrolidinobutyrophenone (HO-MPBP) could be isolated from one liter of CAD58 culture at a yield of approximately 140 µmol.

The aim of the present study was to use a similar approach to biotechnologically synthesize *N*-(1-phenylcyclohexyl)-3-hydroxypropanamine. As shown in Fig. 1, this is a common metabolite of the phencyclidine-derived drugs *N*-(1-phenylcyclohexyl)-3-ethoxypropanamine (PCEPA) and *N*-(1-phenyl-cyclohexyl)-3-methoxypropanamine (PCMPA) and hence might either be considered as *O*-deethyl-PCEPA or *O*-demethyl-PCMPA. The respective *O*-dealkylation steps are catalyzed by the same CYP isoenyzmes, namely CYP2B6, CYP2C19, CYP2D6, and CYP3A4. In the present study PCEPA was used as parent drug and CYP2D6 heterologously expressed in fission yeast strain CAD58 was used as model enzyme.

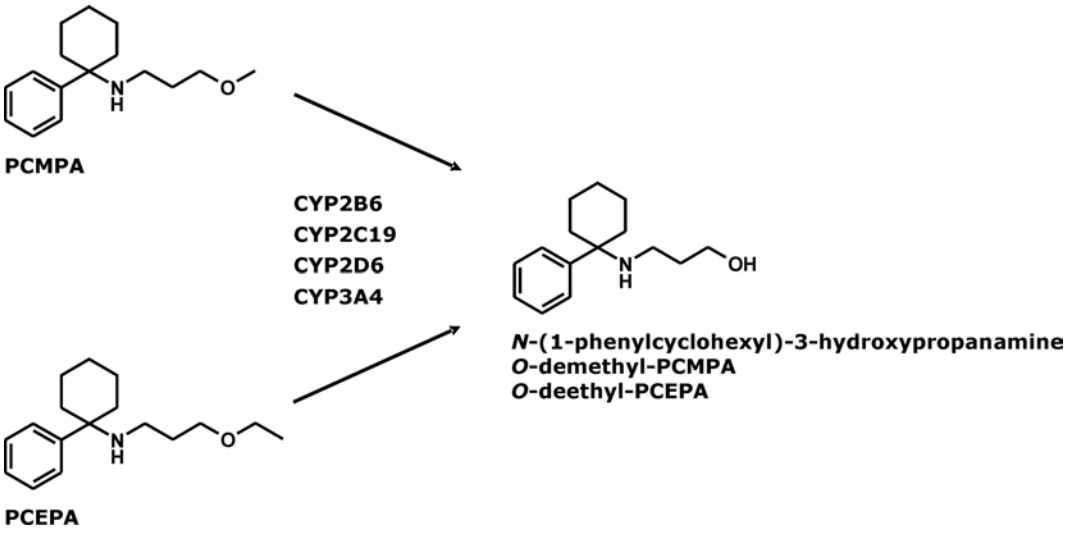

Figure 1 *O*-dealkylation of PCMPA and PCEPA to *N*-(1-phenylcyclohexyl)-3-hydroxypropan-amine by the given CYP isoenzymes.

2. Experimental

2.1 Chemicals and reagents

PCEPA·HCl was provided from Hessisches Landeskriminalamt (Wiesbaden, Germany) for research purposes. Varian Bond Elut SCX HF cartridges (5 g, 2 ml) were obtained from Varian, Darmstadt (Gernamy). Fission yeast strain CAD58 heterologously expressing human CYP2D6 was constructed as described elsewhere [1] and provided by PomBioTech GmbH (Saarbrücken, Germany). All chemicals were from Merck (Darmstadt, Germany) and were of analytical grade.

2.2 Biotechnological synthesis of *O*-deethyl-PCEPA

For biotechnological synthesis of *O*-deethyl-PCEPA, 74 mg (250 μmol) of PCEPA·HCl were fermented in a Biostat bioreactor. The fermentation conditions were as follows: CAD58 culture suspended to 2.5×10^8 cells/mL in 1 L of 0.1 mol/L phosphate buffer (pH 8), 30°C, 72 h, 800 rpm, and 1.5 L O_2/min. After incubation, the cells were separated from the supernatants by centrifugation at 8000*g* for 20 min.

2.3 Extraction of the product from the incubation mixture

The incubation supernatant was brought to pH 3.5 with concentrated. H_3PO_4 and submitted to solid-phase extraction (SPE) using Varian Bond Elut SCX HF cartridges (5 g, 20 ml), previously conditioned with 20 mL of methanol and 20 mL of water. After passage of the supernatant, the cartridge was washed with 20 mL of 0.01 M hydrochloric acid and 40 mL of methanol. The product and remaining parent drug were eluted twice with 20 mL freshly prepared mixture of methanol/aqueous ammonia (96:4 v/v). The eluates were evaporated to dryness under reduced pressure and reconstituted in 3.5 ml HPLC solvent (50 mmol/L ammonium formate buffer (pH 3.5):acetonitril (80:20 v/v)).

2.4 Purification of the product using semi-preparative HPLC

Aliquots (250 μL) of the reconstituted solid-phase extract were injected into a Hewlett Packard (HP) Series 1050 HPLC system (Agilent, Waldbronn, Germany) consisting of a pump and a variable wavelength (VWD) detector ($\lambda = 263$ nm) which was coupled to an Advantec SF 2120 Super Fraction collector. The stationary phase was a Merck LiChrospher® RP select column (250×25 mm, 5 μm). The isocratic mobile phase consisted of 50 mmol/L ammonium formate buffer brought to pH 3.5 with formic acid:acetonitrile, 20:80 (v/v). The flow rate was 5 mL/min and the run time was 35 min. The eluent fractions corresponding to the metabolite were collected and combined.

2.5 Isolation of *O*-deethyl-PCEPA as hydrochloride salt

The combined eluent fractions were diluted with water (1:4 v/v) and submitted to solid-phase extraction (SPE) as described above. After elution of the metabolite, with 20 mL freshly prepared mixture of methanol/aqueous ammonia (96:4 v/v), the eluate was evaporated to dryness under reduced pressure and reconstituted in a mixture of chloroform/cyclohexane (1:1, v/v). The solvent was gassed with HCl and afterwards evaporated to a volume of approximately 1 mL. Then 1 mL of cyclohexane was added and after warming at 60°C the mixture was left at 0°C for 2 h. Subsequently, after centrifugation (3000g, 3 min), the supernatant was removed and the precipitated product was twice washed with 1 mL of cyclohexane. For structural confirmation by proton nuclear magnetic resonance (^{1}H-NMR), solutions of PCEPA·HCl (10 mg/mL) and of the product (5 mg/L) were prepared in $CDCl_3$. ^{1}H NMR spectra (500 MHz) were recorded on a Bruker DRX 500 at 298 K.

2.6 Analysis of product by GC-MS

The isolated product was derivatized by acetylation as described elsewhere [2] and analyzed using a HP (Agilent Technologies, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5989B MS Engine mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m x 0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280°C; carrier gas, helium; flow-rate 1 mL/min; column temperature, programmed from 100-310°C at 30°C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode, *m/z* 50-550 u; EI ionization mode: ionization energy, 70 eV, ion source temperature, 220°C; capillary direct interface heated at 260°C. The mass spectrum of the product was compared that of the postulated *O*-deethyl metabolite of PCEPA given in references [2, 3].

2.7 Product purity check by HPLC

For purity check of the isolated *O*-deethyl-PCEPA a HP 1050 series HPLC system consisting of a quarternary pump, a degasser, an autosampler, and a VWD (λ = 263 nm) was used. The stationary phase was a Zorbax® 300-SCX column (2.1×150 mm, 5 µm). The isocroatic mobile phase consisted of 5 mmol/L ammonium formate buffer brought to pH 3 or 3.5 with formic acid:acetonitrile containing 1% (v/v) formic acid, 55:45 (v/v). The flow rate was 0.85 mL/min and the run time was 7 min. Twenty µL of a solution of 1 mg of isolated product in 1 mL of water were injected in the HPLC system. The purity was expressed as the percentage of the product peak area of total peak area.

3. Results and discussion

3.1. Biotechnological synthesis of *O*-deethyl-PCEPA

The aim of the present study was to apply the approach of biotechnological synthesis of drug metabolites using human CYP isoenzymes heterologously expressed in fission yeast to synthesis of *N*-(1-phenylcyclohexyl)-3-hydroxypropanamine, a common metabolite of the phencyclidine-derived designer drugs PCMPA and PCEPA. The fission yeast strain CAD58 heterologously expressing human CYP2D6 was the same that had been used successfully for synthesis of HO-MPBP. Meanwhile, it has been shown that CYP2D6 is the least active of all isoenzymes capable of catalyzing the *O*-dealkylation of these drugs (C. Sauer et al., IATDMCT, Nice 2007). However, at the time of the present study, fission yeast strains expressing any of these other isoenzymes had not yet been available. PCEPA was chosen as parent drug for the metabolic reaction, because in contrast to PCMPA it is not scheduled in the German Controlled Substances Act.

The experimental conditions for the semi-preparative incubation experiments had been evaluated in preliminary experiments showing that pH 8 and 250 µmol of substrate were adequate choices. The finding that a basic pH was favorable for metabolite formation is in good agreement with earlier studies with MPBP [1]. The most probable explanation for this phenomenon is that both PCEPA and MPBP are basic compounds, which can only reach the enzymes inside the fission yeast cells when uncharged, i.e. at a basic pH of the incubation mixture. The cell density in the present study was twice as high as in the earlier study with MPBP, where metabolite formation had increased linearly with cell density up to values of 10^8 cells/mL, the maximum density that could be achieved in an open batch approach. Using a bioreactor as in the present work, this limitation could be overcome.

3.2. Extraction, purification, and isolation of the product

Despite the use of an optimum incubation pH and use of a high cell density only about 20% of the initial PCEPA were *O*-dealkylated after 72 h incubation. This can most probably be explained by the comparatively low catalytic activity of CYP2D6 in this metabolic step. SPE based on the strong cation exchange principle proved very effective for isolation of the metabolite from the incubation mixture. However, the remaining parent drug was also effectively extracted because of its similar physicochemical properties. Therefore, it became necessary to purify the raw product, i.e. the SPE extract. This was achieved by semi-preparative HPLC, which allowed baseline separation of the metabolite, not only from the parent drug but also from co-extracted matrix compounds from the incubation supernatants. The purified product was extracted from the combined eluent fractions by SPE and could be precipitated from

reconstituted extracts as hydrochloride salt. The yield was about 6 mg which corresponds to approximately 8.8% of the theoretic maximum yield. This low yield could possibly be increased by using yeast strains with the more active CYP isoenzymes.

3.3. Characterization of the final product.

For structural confirmation of the final product its ^{1}H-NMR spectrum was compared to that of PCEPA. This comparison showed that the proton signal corresponding to the ethyl group in the spectrum of PCEPA were absent in that of the final product. Moreover, the signals of the protons corresponding to the protons in position 3 of the propanamine moiety showed the characteristic shift expected with the neighboring substituent changing from ethoxy to hydroxy. Seen together, these observations unambiguously proved that the final product was indeed *O*-deethyl-PCEPA. Further analysis by GC-MS showed that the electron ionization mass spectrum of the final product was also identical with mass spectrum attributed to *O*-deethyl-PCEPA based on interpretation of the fragmentation pattern [2, 3]. The final purity check showed that the peak area of the final product corresponded to more than 90% of the total peak area of the UV chromatogram at 263 nm. This demonstrated that the isolated *O*-deethyl-PCEPA was sufficiently pure to be used as reference substance in further experiments on the CYP isoenyzme-dependent metabolism of PCEPA in humans (C. Sauer et al., IATDMCT, Nice 2007).

4. Conclusions

The presented study once again proved the versatility of biotechnological synthesis of drug metabolites using CYP2D6 heterologously expressed in fission yeast. It further demonstrated that semi-preparative HPLC is a very helpful and efficient tool for product purification in the case of incomplete metabolic transformation. Finally, the comparatively low yield of product shows that the choice of the optimum CYP isoenzyme is an important aspect in biotechnological metabolite synthesis and that further experiments with fission yeast strains expressing other CYP isoenzymes are necessary.

5. Acknowledgements

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6. References

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